SPECIFICATION AMENDMENTS

In the paragraph on page 3, lines 2 to 14, please insert the following:

Compositions of the present invention include novel ELR-CXC chemokine antagonist proteins that are capable of binding to CXCR1 or CXCR2 receptors in mammalian inflammatory cells. These include antagonists that are capable of high-affinity binding, wherein "high-affinity" refers to the antagonist's affinity for the receptor being at least about one order of magnitude greater than that of the wild-type chemokine agonist. The novel antagonist proteins also include those that are substantially equivalent (that is, those that contain amino acid substitutions, additions and deletions that do not delete the CXCR1 and CXCR2 binding functions) to a wild-type bovine CXCL8 protein (illustrated herein as the amino acid sequence of SEQ ID NO:2) and also bear a truncation of the first two amino acid residues along with substitutions of Lys11 with Arg and Gly31 with Pro. Analogues of this $CXCL_{(3\text{-}74\text{-}73)}$K11R/G31P are also included, namely $CXCL_{(3\text{-}74\text{-}73)}$K11R/G31P/P32G and $CXCL_{(3\text{-}74\text{-}73)}$K11R/T12S/H13F/G31P. In addition, compounds having a three dimensional structure resulting in high affinity binding to CXCR1 or CXCR2 receptors in mammalian inflammatory cells.

In the paragraph bridging pages 4 and 5, starting at page 4, line 20 to page 5, line 11, please insert the following:

FIG. 1. The G31 P analogue of $CXCL8_{(3\text{-}74\text{-}73)}$K11R is a potent inhibitor of CXCL8-binding to peripheral blood neutrophils. Bovine peripheral blood neutrophils (87-93%purity) were (upper panel) exposed at 4°C for 2 h to $CXCL8_{(3\text{-}73\text{-}74)}$K11R analogues (10 ng/ml) or medium

(med) alone, then washed and similarly incubated with biotinylated CXCL8 (^{biot}CXCL8; 1000 ng/ml or 129 nM). These levels of CXCL8 approximate those found in the lung tissues of animals with pneumonic pasteurellosis (ref. 8, 9). The levels of ^{biot}CXCL8 binding to the cells were determined using ELISA technology. The depicted amino acid substitutions within CXCL8$_{(3-73\underline{3-74})}$K11R included: G31P; P32G; T12S/H13P/G31P; and T12S/H13P/G31P/P32G. The G31P, but not the P32G, analogue was a highly effective antagonist of CXCL8 binding to the cells. With both the G31P and P32G analogues, additional substitutions of T12S and H13F reduced their CXCL8 antagonist activities (lower panel). Neutrophils were exposed simultaneously for 45 min at 4° C to varying concentrations of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P or unlabeled CXCL8 and 20 pM ^{125}ICXCL8. This level of ^{125}I-CXCL8 was chosen as nearly saturating for the cell's high affinity CXCL8 receptors (data not shown). The levels of cell-associated ^{125}I-CXCL8 were assessed using a counter. The data clearly indicate that CXCL8$_{(3-73\underline{3-74})}$K11R/G31P had a substantially higher affinity for the neutrophils than CXCL8.

In the paragraph bridging pages 5 and 6, starting on page 5, line 12 and ending on page 6, line 3, please insert the following:

FIG. 2. CXCL8$_{(3-73\underline{3-74})}$K11R/G31P is not an agonist of neutrophil chemoattraction responses or -glucuronidase release. CXCL8 and the G31P, P32G, or combined G31P/P32G analogues of CXCL8$_{(3-73\underline{3-74})}$K11R were tested for their neutrophil agonist activities, using freshly purified bovine peripheral blood neutrophils. (upper panel) The chemotactic responses to each protein were tested in 30 min microchemotaxis assays and the results expressed as the mean (+/-

SEM) number of cells/40x objective microscope field, as outlined in the methods section. Both the G31P and G31P/P32G analogues displayed little discernable chemotactic activity, while the P32G analogue stimulated substantial responses at 100 ng/ml. (lower panel) The neutrophils were exposed to varying doses of each analogue for 30 min, then the cellular secretion products were assayed for -glucuronidase using the chromogenic substrate p-nitrophenyl--D-glucuronide, as presented in the methods section. The total cellular stores of -glucuronidase were determined from aliquots of cells lysed with Triton-X-100. The enzyme release with each treatment is expressed as the percent of the total cellular stores. None of the analogues had substantial agonist activity, although CXCL8 itself did induce significant enzyme release. The positive control treatment with phorbol-12,13-myristate acetate and calcium ionophore A23187 induced 42+/-6% enzyme release.

In the paragraph on page 6, starting at line 4 and ending at line 17, please insert the following:

FIG. 3 $CXCL8_{(3\text{-}73\underline{3\text{-}74})}$K11R-G31P is a highly effective antagonist ELR-CXC chemokine-medicated neutrophil chemoattraction. The ability of $CXCL8_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P to block chemotactic responses of bovine neutrophils to several ELR-CXC chemokines was measured using 20 min microchemotaxis assays. (left panel) The cells were simultaneously exposed to CXCL8 (1 μg/ml) and varying concentrations of the analogue. The number of cells that responded to the CXCL8 was assessed by direct counting of the chemotaxis assay membranes, as in FIG. 2. $CXCL8_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P was a highly effective competitive

inhibitor of the cell's responses to CXCL8. (middle panel) Dose-response curves for chemoattraction of bovine neutrophils by human CXCL1, CXCL5, or CXCL8. Each chemokine displayed a biphasic activity pattern, with maxima at 1-10 ng/ml and at 1 μg/ml. (right panel) The ability of $CXCL8_{(3-73\underline{3-74})}$K11R/G31P to block the cell's responses to 1 ng/ml of human CXCL5 or CXCL1 or 10 ng/ml of human CXCL8 was assessed as above. $CXCL8_{(3-73\underline{3-74})}$K11R/G31P effectively antagonized each ELR-CXC chemokine, with complete inhibition being achieved with from 3-20 nM $CXCL8_{(3-73\underline{3-74})}$K11R/G31P.

In the paragraph bridging pages 6 and 7 and starting on page 6, line 18 and ending on page 7, line 11, please insert the following:

FIG. 4. $CXCL8_{(3-73\underline{3-74})}$K11R-G31P blocks the activities of CXCL8 and non-CXCL8 chemoattractants expressed within pneumonic airways or in endotoxin-induced mastitis. The effects of monoclonal anti-IL8 antibody 8B6 or $CXCL8_{(3-73\underline{3-74})}$K11R-G31P on neutrophil responses to the chemoattractants expressed within the airways of animals with pneumonic pasteurellosis or in the mammary cisterns of cattle with endotoxin-induced mastitis were assessed as in FIG. 3. (A) Diluted (1:10) bronchoalveolar lavage fluids (BALF) from lesional lung lobes of pneumonic cattle (PNEUMONIA) or teat cistern lavage fluids from cattle with mastitis (MASTITIS) were tested as is (none) or after treatment with either anti-CXCL8 MAb 8B6 (5 μg/ml) or $CXCL8_{(3-73\underline{3-74})}$K11R/G31P (G31P; 1 or 10 ng/ml) for their chemotactic activities compared to medium alone. With both samples, the Mab 8B6 antibodies by themselves neutralized 74% of the chemotactic activities in the samples, while $CXCL8_{(3-73\underline{3-74})}$K11R/G31P reduced the responses by 93-97%. (B) In

order to confirm these results using an alternate strategy, we next absorbed lesional BAL fluids with monoclonal antibody 8B6-immunoaffinity matrices, removing >99% of their content of CXCL8, then tested both their residual chemotactic activities and the ability of $CXCL8_{(3-73\underline{3-74})}$K11R/G31P to antagonize these residual non-CXCL8 chemotactic activities. There was a dose-dependent inhibition of the total and residual chemotactic activities in the samples, indicating that both CXCL8 and non-CXCL8 chemoattractants are expressed in these lesions.

In the paragraph bridging pages 7 and 8 and starting on page 7, line 12 and ending on page 8, line 1, please insert the following:

FIG. 5. $CXCL8_{(3-73\underline{3-74})}$K11R-G31P can ablate endotoxin-induced inflammatory responses in vivo. Two week-old Holstein calves were tested for their neutrophilic inflammatory responses to intradermal endotoxin (1 μg/site) challenge before and at various time after intravenous (i.v.), subcutaneous (subcutan.), or intramuscular (i.m.) injection of $CXCL8_{(3-73\underline{3-74})}$K11R-G31P (75 μg/kg). Fifteen hour endotoxin reaction site biopsies were obtained at 0, 16, 48 and 72 h post-treatment and processed for histopathologic assessment of the neutrophil response, as determined by counting the numbers of neutrophils in nine 40x objective microscope fields per section. (left panel) Photomicrographs of the tissue responses to endotoxin challenge around blood vessels within the reticular dermis prior to (0 h) and 48 h post-treatment. Large numbers of neutrophils accumulated around the vasculature within the reticular dermis in the pre-, but not post-treatment tissues. (B) Graphic presentation of the neutrophil responses to endotoxin challenge either before (0 h) or after (16, 48, 72 h) $CXCL8_{(3-73\underline{3-74})}$K11R-

G31P delivery by each route. ** or ***=p 0.01 or 0.001, respectively, relative to the internal control pretreatment responses.

In the paragraph on page 8, starting at line 2 and ending at line 7, please insert the following paragraph:

FIG. 6 Eosinophils purified from the blood of atopic asthmatic or atopic non-asthmatic donors (left panels) or a subject with a hypereosinophilia (right panel) were assessed for their responses to recombinant human CXCL8, CXCL5, or CCL11, in the presence or absence of the indicated doses of recombinant bovine $CXCL8_{(3-73\underline{3-74})}$K11R/G31P (G31P). Low doses of G31P were able to block the responses of these cells to each of the CXCR1 and CXCR2 ligands, but had no effect on the eosinophil's responses to the unrelated CCR3 ligand CCL11/eotaxin.

In the paragraph on page 8, starting at line 8 and ending at line 11, please insert the following paragraph:

FIG. 7 Neutrophils from the peripheral blood of a healthy donor were tested for their responses to recombinant human CXCL8 or CXCL5 in the presence or absence of bovine $CXCL8_{(3-73\underline{3-74})}$K11R/G31P (G31P; 10 ng/ml). G31P blocked the neutrophil's responses to both ligands.

In the paragraph briding page 8 and page 9, starting on page 8 at line 23 and ending on page 9 at line 12, please insert the following:

When amino terminal truncation of bovine CXCL8 is combined with a lysine to arginine substitution at amino acid 11 (i.e., $CXCL8_{(3-73\underline{3-74})}$K11R), dramatic increases in CXCR1 and CXCR2 receptor affinity are evident, such that $CXCL8_{(3-73\underline{3-74})}$K11R competitively inhibits

the binding of multiple ligands to both receptors (Li, F., and J. R. Gordon. 2001. Biochem. Biophys. Res. Comm. 286:595-600., hereby incorporated by reference. Further truncation into the receptor-signaling ELR motif (e.g., amino acids 4-6 of human CXCL8) of some CXC chemokines can transform them into mild ($CXCL8_{(6-72)}$) to moderate ($CXCL1_{(8-73)}$) receptor antagonists (McColl and Clark Lewis 1999; Moser, B. et al. 1993. J. Biol. Chem. 268:7125-7128). As disclosed herein, the introduction into bovine $CXCL8_{(3-73\underline{3-74})}$K11R of a second amino acid substitution, glycine 31 to a proline residue (i.e., $CXCL8_{(3-73\underline{3-74})}$K11R/G31P), renders this CXCL8 analogue a very high affinity antagonist of bovine and human ELR-CXC chemokine responses. It fully antagonizes the entire array of ELR-CXC chemokines expressed within bacterial or endotoxin-induced inflammatory foci and blocks endotoxin-induced inflammation in vivo.

In the paragraph on page 9, starting at line 13 and ending at line 17, please insert the following paragraph:

Although the following discussion deals primarily with bovine neutrophils, other mammalian (including human) inflammatory cells also display CXCR1 and CXCR2 receptors (see, for example, Benson, M. et al. 1999. Pediatr. Allergy Immunol. 10:178-185) and so are vulnerable to inhibition by $CXCL8_{(3-73\underline{3-74})}$K11R/G31P. Accordingly, the present invention has broad applicability to mammalian ELR-CXC chemokine-mediated pathologies.

In the paragraph on page 11, starting at line 1 and ending at line 19, please insert the following paragraph:

Generation of CXCL8.sub.$_{(3-73\underline{3-74})}$K11R analogues. The high affinity CXCR1/CXCR2 ligand CXCL8.sub.$_{(3-73\underline{3-74})}$K11R, and its

T12S/H13F analogue were generated in accordance with the methods described in Li and Gordon (2001, supra). The Gly31Pro (G31P), Pro32Gly (P32G), and G31P/P32G analogues of these proteins were similarly generated by site-directed mutagenesis using PCR with the appropriate forward and reverse oligonucleotide primers (Table 1). The products from each reaction were digested with DpnI, ligated into the vector pGEX-2T, transfected into HB101 cells, and their sequences verified commercially (Plant Biotechnology Institute, Saskatoon). Briefly, the recombinant bacteria were lysed in the presence of a protease inhibitor cocktail (2 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) and the recombinant fusion proteins in the supernatants purified by affinity chromatography, using glutathione-Sepharose beads in accordance with the methods of Caswell et al. (Caswell, J. L., D. M. Middleton, and J. R. Gordon. 1998. Vet. Immunol. Immunopath. 67:327-340.). The CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R analogues were cleaved from the GST fusion proteins by thrombin digestion, dialysed against phosphate buffered saline (PBS), run through commercial endotoxin-removal columns, and then characterized by polyacrylamide gel electrophoresis (PAGE) and Western blotting with a goat anti-bovine CXCL8 antibody (provided by Dr. M. Morsey). Each purified analogue had a molecular mass of 8 kDa, was specifically recognized by the anti-CXCL8 antibody in the Western blotting, and had a relative purity of 96%, as determined by densitometric analysis of the PAGE gels.

In the paragraph on page 12, starting at line 8 and ending at line 18, please insert the following paragraph:

CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R analogue binding assays. Cells (85-93%

neutrophils) were purified from the blood of cattle in accordance with the Caswell method (Caswell, J. L. et al. 1998. Vet. Immunol. Immunopath. 67:327-340). In preliminary experiments, we determined that none of our analogues affected the viability of neutrophils, as determined by trypan blue dye exclusion. For the broad analogue surveys, neutrophils in HBSS/0.5% BSA were incubated for 2 h at 4°C. with the analogue, washed in cold DMEM, and then incubated for another 2 h at 4°C. with ^{biot}CXCL8 (1000 ng/ml). The cell-associated biotin was detected by incubating the washed cells with alkaline phosphatase-conjugated streptavidin (1:700 dilution) and then with ABTS enzyme substrate. The OD_{405} of the samples was determined using an ELISA plate reader. Medium-treated neutrophils routinely bound sufficient .sup.biotCXCL8 to generate an OD_{405} of 0.5-0.6.

In the paragraph bridging pages 12 and 13, starting on page 12 at line 19 and ending on page 13 at line 6, please insert the following:

For the in-depth studies with CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P, we used ^{125}I-CXCL8 in binding inhibition assays with unlabeled CXCL8 or CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P. In preliminary experiments we determined that the binding equilibrium time of neutrophils for ^{125}I-CXCL8 was 45 min and that 20 pM ^{125}I-CXCL8 just saturated the cell's high affinity receptors. Thus, in our assays, 10^6 purified neutrophils were incubated for 45 min on ice with 20 pM ^{125}I-CXCL8 and varying concentrations of unlabeled competitor ligand. The cells were then sedimented through 6% mineral oil in silicon oil and the levels of cell-associated radio-ligand determined using a counter. The non-specific binding of ^{125}ICXCL8 to the cells was assessed in each assay by including a 200-fold molar excess of

unlabeled ligand in a set of samples. This value was used to calculate the percent specific binding (Coligan, J., A. Kruisbeek, D. Margulies, E. Shevach, and W. Strober. 1994. Current Protocols in Immunology. John Wiley & Sons, New York).

In the paragraph on page 14, starting at line 5 and ending at line 21, please insert the following:

Neutrophil chemotaxis assays. Microchemotaxis assays were run in duplicate modified Boyden microchemotaxis chambers using polyvinylpyrrolidone-free 5 μm pore-size polycarbonate filters, in accordance with known methods (Caswell et al.,1998; Cairns, C. M. et al. 2001. J. Immunol. 167:57-65). For each sample, the numbers of cells that had migrated into the membranes over 20-30 min were enumerated by direct counting of at least nine 40.times. objective fields, and the results expressed as the mean number of cells/40x field (+/- SEM). The chemoattractants included bovine or human CXCL8, human CXCL5 and CXCL1, pneumonic mannheimiosis BALF and mastitis lavage fluids (diluted 1:10-1:80 in HBSS), while the antagonists comprised mouse anti-ovine CXCL8 antibody 8M6 (generously provided by Dr. P. Wood, CSIRO, Australia) or the CXCL8$_{(3\text{-}7\underline{3}3\text{-}\underline{74})}$K11R analogues. In some assays we preincubated the samples with the antibodies (5 μg/ml) for 60 min on ice (Gordon, J. R. 2000. Cell Immunol. 201:42-49). In others we generated CXCL8-specific immunoaffinity matrices with the 8M6 antibodies and protein-A-Sepharose beads and used these in excess to absorb the samples (Caswell et al.,1997; Gordon, J. R., and S. J. Galli. 1994. J. Exp. Med. 180:2027-2037); the extent of CXCL8 depletion was confirmed by ELISA of the treated samples. For assays with the

recombinant antagonists, the inhibitors were mixed directly with the samples immediately prior to testing.

In the paragraph on page 15, starting at line 5 and ending at line 19, please insert the following:

CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P blockade of endotoxin responses in vivo. We used a sequential series of 15 h skin tests to test the ability of CXCL8$_{(3\text{-}73)}$K11R/G31P to block endotoxininduced inflammatory responses in vivo. For each test, we challenged 2 week-old healthy Holstein cows intradermally with 1 μg endotoxin in 100 μl saline, then 15 h later took 6 mm punch biopsies under local anaesthesia (lidocaine) and processed these for histopathology (Gordon and Galli, 1994). Following the first (internal positive control) test, we injected each animal subcutaneously, intramuscularly, or intravenously with CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P (75 μg/kg) in saline, then challenged them again with endotoxin, as above. The animals were challenged a total of 4 times with endotoxin, such that 15 h reaction site biopsies were obtained at 0, 16, 48, and 72 h post-treatment. The biopsies were processed by routine methods to 6 .mu.m paraffin sections, stained with Giemsa solution, and examined in a blinded fashion at 400- magnification (Gordon and Galli, 1994; Gordon, J. R. 2000. J. Allergy Clin. Immunol. 106:110-116). The mean numbers of neutrophils per 40x objective microscope field were determined at three different depths within the skin, the papillary (superficial), intermediate, and reticular (deep) dermis.

In the paragraph on page 17, starting at line 3 and ending at line 18, please insert the following:

CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P competitively inhibits CXCL8

binding to neutrophils. We surveyed the ability of each CXCL8$_{(3-73\underline{3-74})}$K11R analogue to bind to the CXCL8 receptors on neutrophils, and thereby compete with CXCL8 as a ligand. In our initial surveys, we employed ^{biot}CXCL8 binding inhibition assays, incubating the cells with the analogues (10 ng/ml) for 2 h at 4°C. prior to exposure to ^{biot}CXCL8 (1 μg/ml). This level of CXCL8 approximates those found in the lung tissues of sheep with experimental pneumonic mannheimiosis (Caswell, J. L. 1998. The role of interleukin-8 as a neutrophil chemoattractant in bovine bronchopneumonia. Ph.D. thesis, Department of Veterinary Pathology, University of Saskatchewan). We found that CXCL8$_{(3-73\underline{3-74})}$K11R/G31P was a potent antagonist of CXCL8 binding in this assay (FIG. 1), such that 10 ng/ml of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P blocked 95% of subsequent ^{biot}CXCL8 binding to the cells. When tested at this dose, CXCL8$_{(3-73\underline{3-74})}$K11R/P32G blocked only 48% of CXCL8 binding, while unlabeled CXCL8 itself competitively inhibited 30% of ^{biot}CXCL8 binding. Introduction into CXCL8$_{(3-73\underline{3-74})}$K11R/G31P or CXCL8$_{(3-73\underline{3-74})}$K11R/P32G of additional amino acid substitutions at Thr12 and His13 substantially reduced the antagonist activities of the analogues (FIG. 1). This data clearly suggests that pre-incubation of neutrophils with CXCL8$_{(3-73\underline{3-74})}$K11R/G31P strongly down-regulates subsequent binding of CXCL8.

In the paragraph bridging pages 17 and 18, starting on page 17 at line 19 and ending on page 18 at line 2, please insert the following paragraph:

In order to more finely map the ability of CXCL8$_{(3-73\underline{3-74})}$K11R/G31 to inhibit the binding of CXCL8, in our next set of experiments we simultaneously exposed the cells to ^{125}ICXCL8 and

varying doses of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P or unlabeled CXCL8. We found that CXCL8$_{(3-73\underline{3-74})}$K11R/G31P was about two orders of magnitude more effective than wildtype CXCL8 in inhibiting the binding of 20 pM ^{125}I-CXCL8 to the cells (FIG. 1). The concentration for inhibiting 50% of labeled ligand binding (IC_{50}) was 120 pM for unlabelled CXCL8, and 4 pM for CXCL8$_{(3-73\underline{3-74})}$K11R/G31P. This data suggests that CXCL8$_{(3-73\underline{3-74})}$K11R/G31P is a very potent competitive inhibitor of CXCL8 binding to neutrophils.

In the paragraph on page 18, starting at line 3 and ending at line 18, please insert the following:

CXCL8$_{(3-73\underline{3-74})}$K11R/G31P does not display neutrophil agonist activities. While CXCL8$_{(3-73\underline{3-74})}$K11R/G31P was certainly a high affinity ligand for the neutrophil CXCL8 receptors, it would equally well do so as an agonist or an antagonist. Thus our next experiments addressed the potential agonist activities of the CXCL8$_{(3-73\underline{3-74})}$K11R analogues we generated, as measured by their abilities to chemoattract these cells or induce release of the neutrophil granule hydrolytic enzyme -glucuronidase in vitro (FIG. 2). We found that even at 100 ng/ml, CXCL8$_{(3-73\underline{3-74})}$K11R/G31P was a poor chemoattractant, inducing 13.9+/-4% or 5.4+/-2% of the responses induced by 1 or 100 ng/ml CXCL8 ($p<0.001$), respectively. At 100 ng/ml, the CXCL8$_{(3-73)}$K11R/P32G analogue induced a response that was fairly substantial (38.3+/-2% of the CXCL8 response), while the combined CXCL8$_{(3-73\underline{3-74})}$K11R/G31P/P32G analogue also was not an effective chemoattractant. When we assessed their abilities to induce -glucuronidase release, we found that none of the CXCL8$_{(3-73\underline{3-74})}$K11R analogues was as effective as CXCL8 in inducing

mediator release. Indeed, we found only background release with any of them at 10 ng/ml, and at 100 ng/ml only CXCL8$_{(3-73\underline{3-74})}$K11R/G31P/P32G induced significant neutrophil responses (FIG. 2). Given the combined CXCL8 competitive inhibition and neutrophil agonist data, from this point on we focused our attention on CXCL8$_{(3-73\underline{3-74})}$K11R/G31P.

In the paragraph bridging pages 18 and 19, starting on page 18 at line 20 and ending on page 19 at line 5, please insert the following:

CXCL8$_{(3-73\underline{3-74})}$K11R/G31P blocks neutrophil chemotactic responses to both CXCR1 and CXCR2 ligands. The most pathogenic effect of inappropriate ELR$^+$ chemokine expression is the attraction of inflammatory cells into tissues. Thus, we next assessed the impact of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P on the chemotactic responses of neutrophils to high doses of CXCL8 (FIG. 3). As predicted from our in vivo observations in sheep and cattle (33), 1 μg/ml (129 nM) CXCL8 was very strongly chemoattractive, but even very low doses of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P ameliorated this response. The addition of 12.9 pM CXCL8$_{(3-73\underline{3-74})}$K11R/G31P reduced the chemotactic response of the cells by 33%. The IC_{50} for CXCL8$_{(3-73\underline{3-74})}$K11R/G31P under these conditions was 0.11 nM, while complete blocking of this CXCL8 response was achieved with 10 nM CXCL8$_{(3-73\underline{3-74})}$K11R/G31P.

In the paragraph on page 9, starting at line 7 and ending at line 19, please insert the following:

When we tested the efficacy of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P in blocking responses to more subtle bovine CXCL8 challenges, we also extended the study to assess the ability of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P to block neutrophil responses to human CXCL8 as well as to the human

CXCR2-specific ligands CXCL1 and CXCL5. Each of these is expressed in the affected tissues of pancreatitis (Hochreiter, W. W. et al. 2000. Urology. 56:1025-1029) or ARDS (Villard et al., 1995) patients at 1-10 ng/ml. We found that bovine neutrophils were responsive to 1 ng/ml hCXCL1 or hCXCL5, and similarly responsive to 10 ng/ml hCXCL8 (FIG. 3), so we employed these doses to test the effects of $CXCL8_{(3-73\underline{3-74})}$K11R/G31P on neutrophil responses of these ligands. The neutrophil responses to hCXCL1 and hCXCL5 were reduced to 50% by 0.26 and 0.06 nM $CXCL8_{(3-73\underline{3-74})}$K11R/G31P, respectively, while their responses to hCXCL8 were 50% reduced by 0.04 nM $CXCL8_{(3-73\underline{3-74})}$K11R/G31P (FIG. 3). This data indicates that $CXCL8_{(3-73\underline{3-74})}$K11R/G31P can antagonize the actions of multiple members of the ELR-CXC subfamily of chemokines.

In the paragraph bridging pages 19 and 20, starting on page 19 at line 21 and ending on page 20 at line 10, please insert the following:

$CXCL8_{(3-73\underline{3-74})}$K11R/G31P is an effective in vitro antagonist of the neutrophil chemokines expressed in bacterial pneumonia or mastitis lesions. We wished to test the extent to which our antagonist could block the array of neutrophil chemoattractants expressed within complex inflammatory environments in vivo. Thus, we chose two diseases in which chemokine-driven neutrophil activation contributes importantly to the progression of the pathology, mastitis and pneumonic mannheimiosis. We utilized an endotoxin model of mastitis (Persson, K. et al., 1993. Vet. Immunol. Immunopathol. 37:99-112), in which we infused 5 μg of endotoxin/teat cistern and 15 h later lavaged each cistern. Neutrophils comprised 82 and 6%, respectively, of the cells from endotoxin and saline-control cisterns, with the bulk of the remaining cells comprising

macrophages. The diluted (1:10) wash fluids induced strong in vitro neutrophil chemotactic responses, and the addition of anti-CXCL8 antibodies to the samples maximally reduced these by 73+/-8% (FIG. 4A), relative to the medium control. On the other hand, the addition of 1 ng/ml of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P to the samples reduced their chemotactic activity by 97+/-3%.

In the paragraph on page 20, starting at line 11 and ending at line 18, please insert the following:

Neutrophils also comprised 93+/-12% of the cells recovered from the BALF of cattle with advanced pneumonic mannheimiosis. When tested in vitro, these samples too were strongly chemotactic for neutrophils, and the addition of anti-CXCL8 antibodies maximally reduced their neutrophil chemotactic activities by 73+/-5% (FIG. 4A). Treatment of these BALF samples with 1 or 10 ng/ml of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P reduced the neutrophil responses by 75+/-9 or 93+/-9%, respectively, relative to the medium controls. This data suggests that CXCL8$_{(3-73\underline{3-74})}$K11R/G31P blocks the actions of CXCL8 and non-CXCL8 chemoattractants in these samples.

In the paragraph bridging page 20, starting on page 20 at line 19 and ending on page 21 at line 8, please insert the following:

In order to confirm these observations using an alternate strategy, we next depleted bacterial pneumonia BALF samples of CXCL8 using immunoaffinity matrices, then assessed the efficacy of CXCL8$_{(3-73\underline{3-74})}$K11R/G31P in blocking the residual neutrophil chemotactic activities in the samples (FIG. 4B). The untreated lesional BALF samples contained 3,215+/-275 pg/ml CXCL8, while the immunoaffmity-absorbed BALF

contained 24+/-17 pg/ml CXCL8. In this series of experiments the neutrophil response to the CXCL8-depleted BALF samples was 65.4+/-4% of their responses to the unabsorbed samples. It is known that CXCL8 can contribute as little as 15% of the neutrophil chemotactic activities in pneumonic mannheimiosis BALF obtained from an array of clinical cases (Caswell et al., 2001). Whereas the CXCL8 depletion treatments were 99% effective in removing CXCL8, there remained in these samples substantial amounts of neutrophil chemotactic activities, and the addition of 1 ng/ml CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P fully abrogated their cumulative effects (FIG. 4B). This data unequivocally confirmed that CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P also antagonizes the spectrum of non-IL-8 chemoattractants expressed in these samples.

In the paragraph bridging pages 21 and 22, starting on page 21 at line 10 and ending on page 22 at line 8, please insert the following:

CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P is highly efficacious in blocking endotoxin-induced neutrophilic inflammation in vivo. In our last experiments, we assessed the ability of CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P to block endotoxin-induced inflammatory responses in the skin of cattle, as well as the time-frames over which it was effective. The animals were challenged intradermally with 1 μg bacterial endotoxin 15 h before (internal positive control response), or at three different times after, intravenous, subcutaneous or intramuscular injection of CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P (75 μg/kg). Thus, punch biopsies of 15 h endotoxin reaction sites were taken 15 min before treatment and at 16, 48 and 72 h after injection of the antagonist into each animal, and the numbers of infiltrating neutrophils were determined in a blinded fashion for the

papillary (superficial), intermediate and reticular dermis of each biopsy. Prior to the antagonist treatments, strong neutrophilic inflammatory responses were evident at the endotoxin challenge sites in each animal (FIG. 5). Within the biopsies, the responses in the papillary dermis were mild in all animals (data not shown) and became progressively more marked with increasing skin depth, such that maximal inflammation (neutrophil infiltration) was observed around the blood vessels in the reticular dermis (FIG. 5A). Following the CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P treatments, the inflammatory responses observed within the 16 h biopsies were 88-93% suppressed, while those in the 48 h biopsies were 57% (intravenous) to 97% (intradermal) suppressed, relative to their respective pretreatment responses. By 72 h post-treatment the effects of the intravenously administered antagonist had worn off, while the endotoxin responses in the intradermally and subcutaneously treated cattle were still 60% suppressed. This data clearly indicates that CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P is a highly effective antagonist of endotoxin-induced inflammatory responses in vivo, that these effects can last for 2-3 days, and that the route of delivery markedly affects the pharmacokinetics of this novel antagonist.

In the table heading on page 23, lines 6 to 9, please insert the following:

CXCL8(3-73<u>3-74</u>)K11R ANALOGUE

In the paragraph on page 24, starting at line 1 and ending at line 12, please insert the following:

We demonstrated herein that CXCL8$_{(3\text{-}73\underline{3\text{-}74})}$K11R/G31P is a high affinity antagonist of multiple ELR-CXC chemokines. In vitro, this

antagonist effectively blocked all of the neutrophil chemotactic activities expressed in mild to intense inflammatory lesions within two mucosal compartments (lungs, mammary glands), and up to 97% blocked endotoxin-induced inflammatory responses in vivo. We identified CXCL8 as a major chemoattractant in the pneumonia and mastitis samples, but also demonstrated that 35% of the activity in the bacterial pneumonia samples was due to non-CXCL8 chemoattractants that were also effectively antagonized by $CXCL8_{(3-73\underline{3-74})}$K11R/G31P. Based on studies of inflammatory responses in rodents (Tateda et al., 2001; Tsai et al., 2000), cattle (Caswell et al., 1997), and humans (Villard et al., 1995), it is clear that these samples could contain numerous ELR^+ CXC chemokines (e.g., CXCL5, and CXCL8) to which $CXCL8_{(3-73\underline{3-74})}$K11R/G31P has an antagonistic effect.